Transcriptome Profiling Revealed Candidate Genes, Pathways and Transcription Factors Related to Nitrogen Utilization and Excessive Nitrogen Stress in Perennial Ryegrass

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Research Article

Keywords: Perennial ryegrass, transcriptome, nitrogen utilization, excessive nitrogen stress

Posted Date: June 15th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-599176/v1

Version of Record: A version of this preprint was published at Scientific Reports on March 1st, 2022. See the published version at https://doi.org/10.1038/s41598-022-07329-7.

Abstract

Ryegrass (Lolium perenne L.) is a type of the high quality forage grasses, which can be a good nutritional source for herbivorous livestock. However, improving the nitrogen utilization efficiency and avoiding the nitrate toxicity caused by excessive nitrogen have long been the challenging tasks in ryegrass. The molecular response mechanism of ryegrass to nitrogen, especially under the condition of excessive nitrogen, remains unclear.

In this study, the transcription of perennial ryegrass at different nitrogen levels was identified by high-throughput next-generation RNA sequencing. Phenotypic characterizations showed that ryegrass in treatment N0.5 had a better growth state than the other three groups. Treatments N1 and N10 contained excessive nitrogen, which had a stress effect on plant growth. Analysis of differentially expressed genes indicated that 345 and 104 genes are involved in the regulation of nitrogen utilization and excessive nitrogen stress, respectively. GO enrichment analysis revealed that the plant response to nitrogen was mainly enriched in two categories, including “biological process” and “molecular function”. KEGG enrichment analysis suggested that “Photosynthesis-antenna proteins” may respond positively to nitrogen under appropriate nitrogen conditions, whereas “steroid biosynthesis”, “carotenoid biosynthesis” and “C5-branched dibasic acid metabolism” were identified as the top significantly enriched pathways in response to excessive nitrogen. Transcription factor analysis showed that 21 TFs related to nitrogen utilization were classified into 10 transcription factor families, especially the AP2-EREBP and MYB TF families. Four TFs related to excessive nitrogen stress were identified, including LOB, NAC, AP2-EREBP and HB. The expression patterns of these selected genes were also analyzed.

Instruction

Nitrogen is the main nutrient element for plant growth and plays a very important role in promoting crop yield. Increased application of nitrogen fertilizer is one of the necessary measures to achieve high yield. However, excessive application of nitrogen fertilizer not only reduces plant nitrogen utilization efficiency, but also causes a series of problems such as reduced crop planting efficiency, resource waste and environmental pollution (Bijay-Singh, et al. 1995). It is of great significance to study the appropriate application rate of nitrogen fertilizer for the cost-effective and environmentally friendly sustainable development of agriculture. With the development of molecular biology, many genes related to nitrogen utilization have been identified and cloned, which helps to further elucidate the molecular mechanism of nitrogen uptake and utilization in crops, and lays a foundation for improving nitrogen utilization efficiency in crops by means of genetic engineering.

The processes that occur when nitrogen enters plants include absorption, transport and distribution. Two transporter gene families have been implicated in root nitrogen uptake in plants: nitrate transporter 1/small peptide transporter family NPF (NRT1/PTR) and NO3- transporter family (NRT2). The NRT1 and NRT2 families in Arabidopsis thaliana have 53 and 7 genes, respectively. NRT1 was a subset of low affinity NO3- transfer system (LATS), and NRT2 was a component of high affinity NO3- transfer system

(HATS) (Kotur and GLASS 2015). When the external NO3- concentration was lower than 1 mmol·L-1, HATS played a major role in regulating the inward flow of NO3-, and when the external NO3- concentration was greater than 0.5 mmol·L-1, LATS started to play a role(CRAWFORD, N M, et al., 1998). The absorption mechanism of NH4 + by plant roots was similar to that of NO3-, and also has HATS and LATS absorption and transport systems. Among them, the high-affinity absorption and transport system (HATS) of NH4 + depended on the AMT gene family from the root epidermal cells and membrane of the cortex, which are divided into AMT1 and AMT2 subfamilies (Wang, et al. 2018). In addition, four NO3- transport families have been found to be involved in the transport, distribution and utilization of NO3- in plants: NPF (NRT1/pTR) Family, NRT2 family, chloride channel family (CLC), s-type anion channels and their homologues (SLAC/SLAH) (Wang, et al.). Although significant progress has been made in understanding the physiology and molecular biology about nitrogen uptake, transport, distribution and their regulation in plants, the current research mainly focuses on some bulk plants whose performance is greatly affected by the environment, whereas little is known about the molecular mechanism of plant response to nitrogen in other species with relatively narrow application ranges. As for ryegrass, previous studies showed that under salt stress, NRT(nitrate transporter) gene in annual ryegrass seedlings did not respond to moderately low N application(SHAO, A, et al., 2020). The expression of LpNRT1.1 under high nitrogen condition was generally higher than that under low nitrogen condition, suggesting that NRT1.1 may play an important role in LATS(GUO, Q, et al., 2017).

Perennial ryegrass (Lolium perenne L.) is one of the high quality forage grasses, which can be a good nutritional source for herbivorous livestock and promote the development of animal husbandry (Byrne, et al. 2015). However, as a grass family, perennial ryegrass itself does not have the ability to fix nitrogen, and nitrogen for its growth and development comes mainly from the absorption by root systems from the soil. Nitrogen available in the soil is often difficult to meet the needs of forage grass. Therefore, supplementing soil nitrogen with fertilizer is one of the most effective measures for high quality and high yield of forage. It should be noted that more artificial nitrogen fertilizer does not mean the more nitrogen plant can absorb. When the nitrogen application exceeds a certain level, the utilization efficiency of nitrogen will decrease and the nitrate will be enriched in plants, leading to poisoning of grazing livestock by feeding on those with high nitrate content (Wright and J. 1964). Therefore, it is of great importance to study the endogenous molecular mechanism of nitrogen utilization and excessive nitrogen stress in perennial ryegrass, which can provide a theoretical foundation for guiding production practice.

In recent years, studies about nitrogen in ryegrass have focused on its effect on the yield and quality of forage grass, livestock and agricultural by-products. A mini-sward study in Valdivia found the lower the frequency of nitrogen application, the higher the total crude protein content of ryegrass, but the crude protein component was not affected by the application frequency (Loaiza P 2019). Another study showed that applying high levels of nitrogen to a tetraploid or diploid perennial ryegrass meadow, mixed with white clover, effectively increased milk production per hectare per cow (McClearn, et al. 2019). However, the molecular response mechanism of nitrogen in ryegrass is rarely reported.

In this study, we used high-throughput next-generation DNA sequencing (NGS) technologies to identify the transcription of perennial ryegrass at different nitrogen levels to screen candidate genes related to nitrogen utilization and stress and to further understand the molecular mechanism related to nitrogen in ryegrass. Several transcriptomes related to nitrogen in plants have been characterized in rice(Huang, et al. 2016), potato(Tiwari, et al. 2020), Arabidopsis(Peng, et al. 2007), wheat(Ruuska, et al. 2008), sorghum(Gelli, et al. 2017), poplar(Lu, et al. 2019), nankingense(Wang, et al. 2015), and tea(Yang, et al. 2018). However, some of these studies focused on carbon and nitrogen balance at the transcriptional level, and the other part focused on the change of transcriptional level under the condition of nitrogen deficiency. On the basis of previous studies, our study explored changes in the transcription level of ryegrass under the condition of excessive nitrogen, which compensated for the molecular mechanism of plant response to nitrogen to some extent.

Results

Phenotypic characterization of perennial ryegrass at different concentrations of nitrogen

To describe the growth state of perennial ryegrass under different treatments (Figure 1), the plant height, root length, fresh weight, tiller number and chlorophyll content of all samples were determined (Figure 2 A-D, Figure3). The results showed that the plant heights of treatments N0.5 and N1 were significantly higher than those of the other two treatments (Figure 2A). The fresh weight and tiller number of the N0.5 treatment were significantly higher than those of the other treatments (Figure 2C, 2D). However, the root length of treatment N0 as a control was highest and significantly higher than that in the other treatments (Figure 2B). The results of chlorophyll content determination indicated that there was a significant increase in treatment N0.5 compared to the other three groups; meanwhile, a trend of first up and then down was verified among these four treatments (Figure 3). Based on the above, it could be inferred that ryegrass in treatment N0.5 has a better growth state than the other three groups. Treatments N1 and N10 contained excessive nitrogen, which had a stress effect on plant growth.

Sequencing and transcriptome assembly

To comprehensively identify the transcripts of perennial ryegrass in response to nitrogen, 12 cDNA libraries were constructed from four treatments (N0, N0.5, N1, N10) corresponding to the conditions of no nitrogen, moderate amounts of nitrogen, a certain amount of excessive nitrogen and high concentration excessive nitrogen, each of which had three biological replications. In total, 80.58 GB clean data were released, and the content of each sample varied from 6.02 GB to 7.20 GB(table S1) All clean data were subjected to genomic localization analysis (http://185.45.23.197:5080/ryegrassgenome) by HISAT. A minimum of total mapped clean data occupied 61.84% in the relative example, and the proportion of multiple mapped reads or fragments in each example was less than 0.8% (table S2). Through transcriptome assembly, these transcripts were classified into 101376 unigenes, containing 2185 novel

genes(table S3). The raw data was loaded on the SRA database in the NCBI(BioProject ID: PRJNA660099) (table S4).

Analysis of differentially expressed genes (DEGs)

To understand the response of the ryegrass gene to nitrogen, DEGs in three treatments (N0.5, N1 and N10) were screened with treatment N0 as a control. Under these conditions, 883, 1597, 1778 were identified separately as significantly differentially expressed in the treatments N0.5, N1 and N10. In treatment N0.5, 422 genes were upregulated and 461 genes were downregulated compared to the control group. Accordingly, the expression of 546 genes was upregulated and the expression of 1051 genes was downregulated in treatment N1. A total of 642 genes were upregulated and 1166 genes were downregulated in treatment N10 (table S5 A1-2, B1-2, C1-2, Figure 4A-C).

The differential expression of these DEGs was caused by the addition of nitrogen. To identify more genes related specifically to nitrogen utilization, three groups of DEGs were drawn into a Venn diagram (Figure 5a). The results showed that 345 genes are considered to be directly involved in the regulation of nitrogen utilization, among which 322 genes are known and the rest are novel genes(table S6).

In plant growth status identification, the conditions of treatment N0.5 were more suitable for ryegrass growth than the other three groups, and treatment N1 and N10 produced excessive nitrogen stress on the plants. Based on this, we further attempted to screen genes that specifically respond to excessive nitrogen stress. DEGs in treatments N1 and N10 were identified using treatment N0.5 as a control (table S5 D1-2, E1-2, Figure 4 D-E). There were 456 differentially expressed genes (169 upregulated, 287 downregulated) and 494 differentially expressed genes (154 upregulated, 340 downregulated) in treatments N1 and N10. Furthermore, 82 known genes and 22 novel genes were identified in the common genes in treatment N1 and N10 compared to treatment N0.5 (table S7, Figure 5b). These common genes may play an important role in the response of perennial ryegrass to excessive nitrogen stress.

Clustering of unigenes related to nitrogen utilization and excessive nitrogen stress

To gain insights into the expression pattern of those screened unigenes related to nitrogen utilization and excessive nitrogen stress, their expression levels in the different treatments containing different concentrations of nitrogen were clustered separately (Figure 6). Clustering analysis of unigenes related to nitrogen utilization revealed that the expressions of those genes in treatment N0.5 were most similar to those in treatment N1, followed by those in treatment N0, and then those in treatment N10, in general (Figure 6A). Considering the nitrogen content in different treatments, presumably, we could tell that these genes have analogous expression in response to moderate amounts of nitrogen and a certain amount of excessive nitrogen but distinct expression in response to high concentrations of excessive nitrogen. It is worth mentioning that most of the genes related to nitrogen utilization had relatively high expression in

treatment N10, whereas they had relatively low expression in the other three groups. This result suggested that most of them possess similar expression patterns.

On the other hand, there were similar expressions of the genes related to nitrogen excessive nitrogen stress between treatment N0.5 and treatment N0, and something similar happened in treatment N1 and treatment N10 (Figure 6B). It could be inferred that those genes related to nitrogen excessive nitrogen stress are expressed homoplastically under the condition of no nitrogen and moderate amounts of nitrogen, in view of the different nitrogen contents in each treatment. Similarly, these genes had analogous expression in response to excessive nitrogen ranging from a certain concentration to high concentration.

GO and KEGG enrichment analysis of DEGs

To further understand the functional classification and metabolic pathways involved in the response of perennial ryegrass to nitrogen, GO and KEGG enrichment of DEGs was analyzed. We selected 30 GO terms with the most significant enrichment and showed them in the histogram (Figure 7). GO enrichment analysis of DEGs in treatment N0.5 compared to treatment N0 as a control revealed that all selected DEGs have significantly enriched GO terms, which are classified into “biological process” and “molecular function”, respectively. The “oxidation-reduction process” had the maximum enrichment in all “biological processes”. The number of DEGs enriched in “catalytic activity”, “transferase activity”, and “oxidoreductase activity” was also higher than the others in “molecular function” (Figure 7A). This result suggested that the genes of plant response to nitrogen were mainly enriched in “oxidation-reduction process”, “catalytic activity”, “transferase activity” and “oxidoreductase activity” under the conditions of relatively suitable nitrogen. Nevertheless, DEGs in treatment N1 compared to treatment N0.5 as a control only had five significantly enriched GO terms, which were mainly classified into “molecular function” (Figure 7B). We could deduce that more GO terms in “molecular function” are involved in the response mechanism of plants to nitrogen under a certain amount of nitrogen stress. Based on the GO enrichment analysis of DEGs in treatment N10 compared to treatment N0.5 (Figure 7C), there were no significantly enriched GO terms.

Furthermore, DEGs were separately enriched in 60, 37 and 38 pathways (Table S8, S9 and S10). Twenty pathway items with the most significant enrichment were selected for display in Figure 8. DEGs in treatments N0.5 and N1 (Figure 6A, 6B) displayed a certain degree of enrichment in the “nitrogen metabolism” pathway. The results in treatment N0.5 compared to N0 showed that the top enriched pathway of DEGs was “Photosynthesis-antenna proteins”, except “nitrogen metabolism” (Figure 8A). This result suggested that “photosynthesis-antenna proteins” may also respond positively to nitrogen under appropriate nitrogen conditions. Similarly, “steroid biosynthesis” and “carotenoid biosynthesis” were identified as the top two significantly enriched pathways in the KEGG enrichment analysis of DEGs between treatments N1 and N0.5 (Figure 8B). In addition, there were two relatively enriched pathways (“phenylpropanoid biosynthesis”, “phenylalanine metabolism”) with lower Q values (Figure 8B). This

means that genes in these special pathways play an important role in the response mechanism of plants to a certain amount of nitrogen stress. Regarding the pathways enriched in treatment N10 compared to N0.5, “C5-branched dibasic acid metabolism” (only Novel00490|osa:4328147 enriched) and “steriod biosynthesis” pathways showed the top significant enrichment (Figure 8C). It can be indicated that “steriod biosynthesis” may perform some unknown and irreplaceable function under excessive nitrogen stress.

Photosynthesis-antenna proteins, steroid biosynthesis and carotenoid biosynthesis pathways

In KEGG enrichment analysis of DEGs, four special pathways with significantly enriched DEGs were found in three DEG groups caused by the different nitrogen contents. To further explore the response of these pathways to different nitrogen conditions, the enriched DEGs were mapped to those filtered pathways, and their expression was analyzed by clustering severely (Figure 9). There were 9, 2, 2, and 3 DEGs with significant enrichment classified into photosynthesis-antenna proteins, steroid biosynthesis and carotenoid biosynthesis pathways. These DEGs enriched in three special pathways displayed relatively broad expression patterns. Notably, the different DEGs in treatment N1 and N10 compared to treatment N0.5 were significantly enriched in the steroid biosynthesis pathway simultaneously. It could be indicated that the diverse parts of the steroid biosynthesis pathway function in the perennial ryegrass response to excessive nitrogen ranging from a certain concentration to a high concentration.

Nitrogen metabolism pathway

By KEGG enrichment analysis, we found 15 DEGs related to the right amount of nitrogen in ryegrass, which had homology to the previously identified genes in nitrogen metabolism pathway(table S8). 12 of them were successfully matched in the set of genes(table S6) we screened as genes related to nitrogen utilization. This was due to the different methods of statistical analysis of data. In the same way, 1 DEG and 0 DEG related to a certain amount of nitrogen stress and high concentration of nitrogen stress, respectively, performed homology to the previously identified genes in nitrogen metabolism pathway(table S9 and S10). This DEG was not included in the gene collection we screened as genes related to excessive nitrogen stress(table S7). Considering that we screened 345 and 104 DEGs as candidate genes related to nitrogen utilization and excessive nitrogen stress, respectively(table S6 and S7), it was reasonable to assume that the remaining 437 DEGs were genes related to the nitrogen response specifically in ryegrass, excluding the 12 DEGs enriched in the nitrogen metabolism pathway.

The expression analysis of these 12 DEGs showed that they performed a similar expression pattern, which was up-regulated in N0 treatment and down-regulated in other treatments, except

ref0006917-exonerate_est2genome-gene-0.0 with the reverse trend(Figure 10A). This indicated that ref0006917-exonerate_est2genome-gene-0.0 might play a positive role in nitrogen utilization, while the

remaining genes performed some important functions in the absence of nitrogen.

Nitrate transporter family(NRT)

A total of 209 members of this transcriptome that were homologous to the NRTs in *Arabidopsis* were extracted, and 104 of them were differentially expressed in each treatment(table S11). The expression patterns of these differentially expressed genes varied, but they were more closely expressed in N0.5, N1, and N10 treatments than in N0 treatments(Figure 10B). This suggested that these NRTs might perform different functions in the absence of nitrogen than in the presence of nitrogen.

Identification of transcription factors

There is accumulating evidence that transcription factors (TFs) play key roles in various regulatory mechanisms(Hu, et al. 2017). Therefore, transcription factors from DEGs were identified for the purpose of further studying the molecular regulatory network of perennial ryegrass in response to nitrogen (Figure 11A). In total, 185 TFs, among which there were 14 novel genes, were distinguished in all differentially expressed genes. These TFs belonged to 37 transcription factor families, including the top three TF families (AP2-EREBP, MYB, NAC) with quantities greater than ten.

The expression patterns of these TFs changed in a relatively large range. Based on this, TFs related to nitrogen utilization and excessive nitrogen stress were characterized (Figure 11B, 11C). In total, 21 TFs related to nitrogen utilization, among which AP2-EREBP and MYB TF families occupied the largest number, were classified into 10 transcription factor families (Figure 10B). These TFs exhibited semblable expression in treatments N0.5, N1 and N10, and most of them possessed similar expression patterns in all treatments. Accordingly, 4 TFs related to excessive nitrogen stress were identified, which belonged to 4 transcription factor families, including LOB, NAC, AP2-EREBP and HB (Figure 11C). The expression of these TFs was relatively similar in treatments N1 and N10, and 2 TFs in the HB transcription factor family had similar expression patterns. Remarkably, ref0030344-processed-gene-0.2 from the AP2-EREBP transcription factor family concurrently exhibited a positive response to nitrogen absorption and excessive nitrogen stress.

Expression validation by qRT-PCR

To verify the accuracy of expression from RNA-Seq, 22 unigenes with significantly different expression levels were randomly selected for qRT-PCR analysis. These unigenes, among which 12 genes from DEGs related to nitrogen utilization and 10 from excessive nitrogen stress, contained 8 transcription factors and 14 functional genes. Almost all expression patterns of these genes were accordant in qRT-PCR analysis compared to those in the transcriptome profiling (Figure 12). Thus, the results of qRT-PCR with good

coherence to those of RNA-Seq contributed to reflecting the high credibility and accuracy of transcriptome data.

Discussion

The response of plants to nitrogen as a key nutrient element for plant growth and development involves a complex regulatory network involving various kinds of functional genes and transcription factors. Illuminating these intricate molecular regulatory mechanisms would make great contributions to more scientific nitrogen applications in production practice. In this study, to produce added insight into the mechanisms of perennial ryegrass response to nitrogen, we paid more attention to those induced by nitrogen utilization and excessive nitrogen stress. The transcripts of perennial ryegrass cultivated at different concentrations of nitrogen were analyzed by RNA-Seq, mainly for the identification of differentially expressed genes related to nitrogen utilization and excessive nitrogen stress.

Phenotypic characterization of perennial ryegrass

Under the condition of moderate amounts of nitrogen, the plants had relatively larger plant heights, fresh weights and tiller numbers than those under other conditions (Figure 2A, C, D). On the other hand, it has been suggested, to some degree, that 0.5 times the standard hoagland nutrient solution in treatment N0.5 provides the plants with a relatively appropriate amount of nitrogen indeed. Analogously, there is evidence that increasing nitrogen supply has led to increasing plant biomass in a nitrogen-deficient soil environment. For example, the biomass of sugarcane leaves can have been significantly increased along with increased nitrogen fertilizer(Jian, et al. 2011). Compared with the low nitrogen condition, paper birch in all groups under the high nitrogen condition grew faster, their total biomass was larger, and their root/shoot ratio was lower. However, relatively larger root lengths were found in the no nitrogen treatment group than in the other groups (Figure 2B). Existing research suggests that plants invest more carbon assimilating substances into the root system to promote root growth and development to obtain more restricted resources, resulting in an increase in the root/shoot ratio of plants when soil nutrients are scarce(Jian, et al. 2011). Regarding the chlorophyll of perennial ryegrass in different concentrations of nitrogen, the content under the condition of 0.5 times the standard hoagland nutrient solution was highest, with a tendency to increase and then decrease in the four treatments (Figure 3). Previous research has shown that under the stress of nitrogen deficiency, the chlorophyll content of plant leaves decreases and the F686 / F740 fluorescence ratio increases(Tartachnyk, et al. 2006). Excessive nitrogen transfer of nutrients in maize has resulted in premature leaf senescence and decreased photosynthetic capacity(Osaki, et al. 1995). Above all, it can be indicated that four kinds of nitrogen concentrations designed to represent the conditions of no nitrogen, moderate amounts of nitrogen, a certain amount of excessive nitrogen and high concentration excessive nitrogen in different treatments are relatively reasonable.

DEGs related to nitrogen utilization and excessive nitrogen stress

A total of 345 screened genes related to nitrogen utilization generally display analogical expression patterns, with rapidly increased expression in cells treated with high concentrations of excessive nitrogen (Figure 6A). A logical speculation that the expression of these genes will increase with the increase in nitrogen supply and their overexpression may cause a certain degree of disorder of nitrogen metabolism in plants under high concentrations of excessive nitrogen stress requires further verification. In addition, the expression patterns of 104 DEGs related to excessive nitrogen stress can be broadly divided into five categories (Figure 6B). This means that these genes may regulate the plant response to excessive nitrogen stress in five corresponding ways. Special pathways that respond positively to different concentrations of nitrogen

The photosynthesis-antenna protein pathway was observed under the condition of moderate amounts of nitrogen in KEGG enrichment analysis. Fifteen DEGs caused by moderate amounts of nitrogen application have been attributed to 2 categories, including CAB1R (osa:4346803) and CAB2R (osa:4324599), which could express two types of light-harvesting complex II chlorophyll a/b binding proteins based on the KEGG database. A previous study showed that OsCSP41b has an impact on leaf colors in rice(Mei, et al. 2017). It can be inferred that these genes are induced by moderate amounts of nitrogen, and some analogous photosynthetic characteristics of perennial ryegrass are displayed in this study. They may act as an important link between nitrogen and plant photosynthetic regulation mechanisms.

DEGs in the carotenoid biosynthesis pathway showed some enrichments under a certain amount of excessive nitrogen, including NCED|osa:4333566 and CCD|osa:4330451. OsNCED3 is involved in seed dormancy, plant growth, abiotic stress tolerance and leaf senescence by regulating the biosynthesis of ABA in rice(Huang, et al. 2018). CitCCD4 converts β-cryptoxanthin and zeaxanthin into β-citraurin, leading to the flavedo of citrus fruit(Gang, et al. 2013). New functions of NCED and CCD selected in this study in excessive nitrogen stress remain to be explored.

The diverse parts of the steroid biosynthesis pathway have been found to function in the perennial ryegrass response to excessive nitrogen ranging from a certain concentration to high concentration, with CYP15G1|osa:4328054, SMT1|osa:4338803 to a certain concentration delta(7)-sterol-C5(6)-desaturase|osa:4325687, probable 3-beta-hydroxysteroid-Delta(8) |osa:4326449, CYP|osa:4328054 to high concentration. Based on previous studies, the application of ammonium sulfate advances the high expression of FPPS, SMT1, SMT2, SMO1, SMO2, ODM and other pathway genes, accompanied by the increased content of sterol required for anilide biosynthesis in Withania somnifera (L.) Dunal(Pal, et al. 2017). CYP51s catalyzes the 14α-demethylation of sterol in all eukaryotes(Cabello-Hurtado, et al. 1997). We can deduce that these genes enriched in the steroid biosynthesis pathway are indeed regulated by exogenous nitrogen, and 14α-demethylation of sterol may be one of the key links between this pathway and excessive nitrogen stress.

A total of 16 DEGs were enriched in nitrogen metabolism pathway, which had homology to the previously identified genes(table S8, S9 and S10). Thereinto, ref0006917-exonerate_est2genome-gene-0.0 performed an unique expression pattern, which was up-regulated as the nitrogen concentration increased. It encoded a kind of carbonic anhydrase, belonging to carbonic anhydrase family(CA). Its main function was to accelerate the interconversion of CO2 to HCO3(CHATTERJEE, J, et al., 2021). This suggested that CAs might be one of the key links regulating carbon and nitrogen balance. On the other hand, DEGs enriched in nitrogen metabolism pathway and classified into nitrate transporter family(NRT) performed closely expression patterns in N0.5, N1, and N10 treatments, compared to N0 treatments. This was also consistent with treatments we set up.

Transcription factors related to nitrogen utilization and excessive nitrogen stress

The AP2-EREBP transcription factor family unique to plants is widely involved in the regulation of plant physiological functions and signal transduction pathways such as salicylic acid, jasmonic acid, abscisic acid and ethylene. AP2-EREBP transcription factors possess a positive response to nitrogen, especially low nitrogen stress. This point has been demonstrated in many species and organs, including sugarcane(Yang, et al. 2019), leaves and roots of watermelon(Nawaz, et al. 2018), maize root(Xiujing, et al. 2016), Arabidopsis thaliana root(Ristova, et al. 2016) and maize(Chen, et al.). In this study, there was also a positive response to moderate amounts of nitrogen and excessive nitrogen stress by identifying these transcription factors. This finding advances the supplementary instruction that the AP2-EREBP transcription factor family plays a functional regulatory role in the plant response to different concentrations of nitrogen. It is worth mentioning that ref0030344-processed-gene-0.2 from the AP2-EREBP transcription factor family was regulated by both nitrogen utilization and excessive nitrogen stress. The function of these TFs in regulating the molecular mechanisms of the plant response to nitrogen deserves to be discussed in the future. MYB transcription factors are one of the largest families of transcription factors in plants and are widely involved in the regulation of the cell cycle(Mu, et al. 2009), secondary metabolism(Liu, et al. 2015), and biotic and abiotic stress(Nakabayashi, et al. 2014). SiMYB3 in Setaria italica plays a key role in regulating root growth, which in turn leads to increased tolerance to low-nitrogen stress(Ge, et al. 2019). Here, four MYB transcription factors have been identified to play some functional implication in nitrogen utilization. This is consistent with our results.

NAC transcription factors not only participate in plant growth and development (such as lateral root development, flower development, secondary wall formation and plant hormone signal transduction). but also play an important regulatory role in plant responses to abiotic stress, such as drought, salinity, water logging and low temperature(Ye, et al. 2018). Some members of the NAC transcription factor family perform great importance in enhancing tolerance to N deficiency in cotton(Magwanga, et al. 2019). In this study, one NAC transcription factor (ref0030817) was found to exercise a regulatory function in excessive nitrogen stress. This may be a new angle in understanding the role of the NAC transcription factor family in excessive nitrogen stress. Something similar happens in LOB and HB transcription factor families

(novel101854,ref0036657). LOB transcription factors are mainly involved in the regulation of plant morphological development and photosynthesis. For example, MtLOB induced by nodule inception regulates lateral root development in *Medicago truncatula*(Schiessl, et al. 2019). However, the potential regulatory mechanisms of LOB transcription factors on excessive nitrogen stress are unclear.

The HB transcription factor is a very important regulatory protein in plants that plays an important regulatory role in plant morphogenesis, hormone response, biological stress and abiotic stress response. BvHb1.2 has high NOD-like activity in seeds, and BvHb1.1 exhibits equal ability to combine NO in nitrate formation to protect chloroplasts from the deleterious effects of NO(Leiva, et al. 2019). In this study, the HB transcription factor (ref0036657) screened to be related to excessive nitrogen stress may be involved in some similar metabolic mechanisms of nitrite in perennial ryegrass, which could be a key point to guide and regulate nitrate accumulation in ryegrass.

Conclusions

At different concentrations of nitrogen, a total of 101376 unigene transcriptional characteristics were collected in perennial ryegrass by RNA-Seq, among which 345 and 104 DEGs were identified to be related to nitrogen utilization and excessive nitrogen stress, respectively.

KEGG enrichment analysis identified four significantly enriched pathways in response to nitrogen at different concentrations, consisting of zeatin biosynthesis, photosynthesis-antenna proteins and isoquinoline alkaloid biosynthesis. A total of 209 members of this transcriptome that were homologous to the NRTs in Arabidopsis were extracted, and 104 of them were differentially expressed in each treatment. In addition, 185 transcription factors from 37 transcription factor families, including the top three TF families (AP2-EREBP, MYB, NAC), were identified as TFs related to nitrogen. Twenty-two transcription factors from 10 transcription factor families, especially the AP2-EREBP and MYB transcription factor families, were considered to perform some important regulatory functions in nitrogen utilization. There were 4 transcription factors related to excessive nitrogen stress, coming from the LOB, NAC, AP2-EREBP and HB transcription factor families. These results provide new insight into the regulatory mechanism of the plant response to nitrogen utilization and excessive nitrogen stress.

Methods

Plant materials

The plant material used in this research was perennial ryegrass "Taya" (variety registration no.:285), which was introduced by professor Liebao Han of Beijing Forestry University from DLF SEEDS A/S Company in Denmark on December 8, 2004. The seeds of perennial ryegrass were preserved at the Lawn Research Institute of Beijing Forestry University (Beijing, China). The collection, preservation and use of plant materials involved in this study complied with relevant institutional, national, and international guidelines and legislation. After a week of vernalization in a 4°C refrigerator, the seeds were placed on

petri dishes with sterile filter paper for germination under natural conditions in the laboratory. After two weeks of seed cultivation, seedlings approximately 5 cm in height were selected and suspended in Hoagland nutrient solution with nitrogen control (Table S12). In a chamber with 16 h light/8 h darkness at 23°C, the seedlings were grown in Hoagland nutrient solution with 0, 0.5, 1, and 10 times the standard concentration of nitrogen for 4 weeks (table S13). The treatments were called N0, N0.5, N1, and N10 in turn. Three plants of each treatment were randomly selected as biological replicates. A total of 12 samples were immediately treated with liquid nitrogen and stored at -80°C.

The determination of phenotypic characterization

Four plants were randomly selected from each treatment as the biological replicates, and their plant height, root length, fresh weight and tiller number were measured. Approximately 0.1 g of each sample was placed in 95% alcohol and then stood in the dark for 24 h, and the specific mass was recorded. The absorbance values of these samples were measured at wavelengths of 665, 649 and 470 nm and were corrected with 95% alcohol. The chlorophyll a/b and total chlorophyll contents were calculated by the corresponding formulas(Wellburn and R. 1994).

RNA isolation and detection

Total plant RNA was extracted using the oxygen RNA extraction kit and then treated with the RNase-Free DNaseI Set to remove contaminating genomic DNA. Four methods were used to detect the quality of RNA as follows. Agarose gel electrophoresis was used to analyze the degradation degree and contamination of RNA. The purity of RNA was detected by Nanodrop (od260/280 ratio). RNA concentration was accurately quantified by Qubit. The integrity of RNA was precisely detected by Agilent 2100.

Library construction for transcriptome sequencing

After the sample passed the test, the mRNA of the samples was enriched by magnetic beads with Oligo (dT) binding with the polyA tail of mRNA through a-t complementary pairing. Second, mRNA was fragmented into short segments using fragmentation buffer. Using mRNA as a template, a strand of cDNA was synthesized using random hexamers, and then two strand cDNA was synthesized using buffer, dNTPs and DNA polymerase I. AMPure XP beads were used to purify double strand cDNA. Purified double-stranded cDNA was repaired at the end, followed by adding an A tail and connecting sequencing joints. AMPure XP beads were used to select each segment size. Finally, PCR enrichment was carried out to obtain the final cDNA library.

After the construction of the library, Qubit2.0 was used for initial quantification, and the library was diluted to 1 ng/μl. Then, the insert size of the library was detected by Agilent 2100. After the insert size

met the expectation, qPCR was used to accurately quantify the effective concentration of the library (> 2 nM) to ensure the quality of the library.

HiSeq sequencing was carried out after pooling different libraries in accordance with the requirements of effective concentration and target disembarkation data.

Quality control, read mapping and functional annotation

The original image data files obtained by high-throughput sequencing (such as Illumina HiSeqTM) were transformed into Sequenced Reads, called raw data, by CASAVA Base Calling analysis. The error rate of each base sequencing was converted by sequencing the Phred value (Phred score, Qphred) using the formula (formula 1: Qphred = -10log10(e)). The segregation of bases A/T and C/G was confirmed by a base C/G content distribution test. To ensure the quality of information analysis, we filtered out low-quality reads with connectors from raw data and obtained new data called clean reads for further analysis.

HISAT software was selected to localize the filtered sequences. The software default parameters are used during the analysis. Clean reads were compared to the reference genome(http://185.45.23.197:5080/ryegrassgenome). Read distribution in different regions of the reference genome was counted, including those that align to exon occupied the highest content and intron derived from pre-mRNA residues and intron retention during alternative splicing. The density of total mapped reads mapped to each chromosome in the genome (plus or minus chains) was analyzed. Integrative Genomics Viewer was used to visually browse the bam files containing RNA-seq reads for genomic alignment and corresponding reference genome and annotation files.

Analysis of differentially expressed genes (DEGs)

It adopted DESeq(Anders, et al. 2015) to normalize read count first and the software default parameters are used, and then the hypothesis testing probability (p-value) was calculated according to the model. Finally, multiple hypothesis testing and correction were performed to obtain the value of FDR (error detection rate). The screening criteria for differentially expressed genes were pval < 0.05 and |log2(FoldChange)| > 1. The overall distribution of differential genes was inferred from the volcano map. The number of differentially expressed genes and the overlap between the comparison groups are shown by using the Venn diagram. The FPKM value of the differentially expressed genes under different experimental conditions was taken as the expression level for hierarchical clustering analysis. The relative expression level log2(ratios) of differentially expressed genes were clustered by h-cluster, k-means and SOM. Different clustering algorithms divide the differential genes into several clusters, and the genes in the same cluster have similar expression level variation trends under different processing conditions.

GO and KEGG enrichment analysis of differentially expressed genes

GOseq(Young, et al. 2010) was the software method used in the GO enrichment analysis, which was based on Wallenius non-central geometric distribution. GO terms with corrected P values less than 0.05 were considered significantly enriched by differentially expressed genes.

On the other hand, the pathway in the KEGG database was taken as the unit, and a hypergeometric test was applied to determine the pathway significantly enriched in differentially expressed genes compared with the whole genomic background. We used KOBAS software to test the statistical enrichment of differentially expressed genes in KEGG pathways.

Transcription factor analysis

ITAK software was used to predict transcription factors in differentially expressed genes.

The TF (transcription factor) family and rules defined in the database were used to identify TF by hmmscan(Paulino, et al. 2009).

Quantitative real-time PCR analysis

Twenty-four unigenes were selected for quantitative real-time PCR analysis to verify the accuracy of the transcriptome data and the actual expression of related genes. The previously extracted total RNA was reverse transcribed into cDNA as a qPCR template. All primers designed with Primer Premier 5.0 software are displayed in table S14. *EIF4A* gene from ryegrass was used as an internal reference gene to calculate the relative expression level(LEE, J M, et al., 2010). Quantitative real-time PCR analysis was performed by a Bio-Rad CFX connected server system. Reaction conditions were set as follows: 95°C for 10 min; 95°C for 15 s, 60°C for 1 min, 40 cycles. Each reaction was completed with four technical replicates. The relative expression levels of all genes were determined by the 2-deltadelta Ct method(Livak and Schmittgen 2000).

Abbreviations

TFs: transcription factors

KEGG: Kyoto Encyclopedia of Genes and Genomes

GO: Gene Ontology

DEGs: differentially expressed genes

qRT-PCR: Quantitative Real-Time PCR

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets generated and/or analyzed during the current study will be available in the NCBI database in Aug. 1st, 2021.

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

The program was supported by the National Natural Science Foundation of China (No. 31971770) and Beijing Natural Science Foundation (No.6204039). Both of them provide funding for the entire research effort, which is used for material maintenance, reagents, data collection, labor costs and so on.

Author contribution

Yinruizhi Li proposed ideas and designed the experiments. Yinruizhi Li and Mengdi Wang completed the experiments. Yinruizhi Li and Ke Teng analyzed the experimental data. Di Dong, Zhuocheng Liu and Tiejun Zhang proposed key opinions that cannot be ignored. Yinruizhi Li and wrote the manuscript. Liebao Han played a guiding role in the whole work process. All authors read and reached an agreement with the final manuscript.

Acknowledgements

Not applicable

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Figures

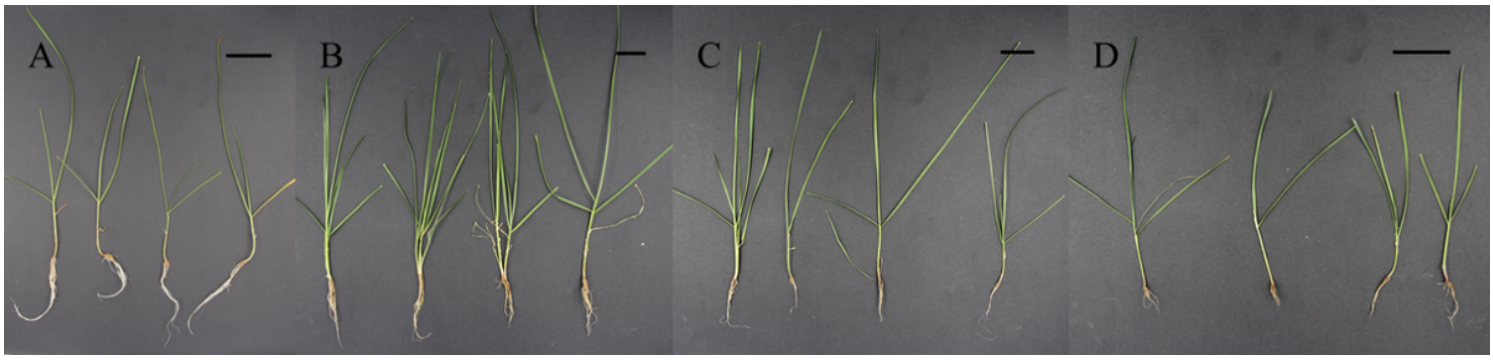

Figure 1

The growth state of perennial ryegrass under different treatments. A-D represent treatments N0, N0.5, N1, and N10, respectively. The black scale in each figure indicates 3 cm.

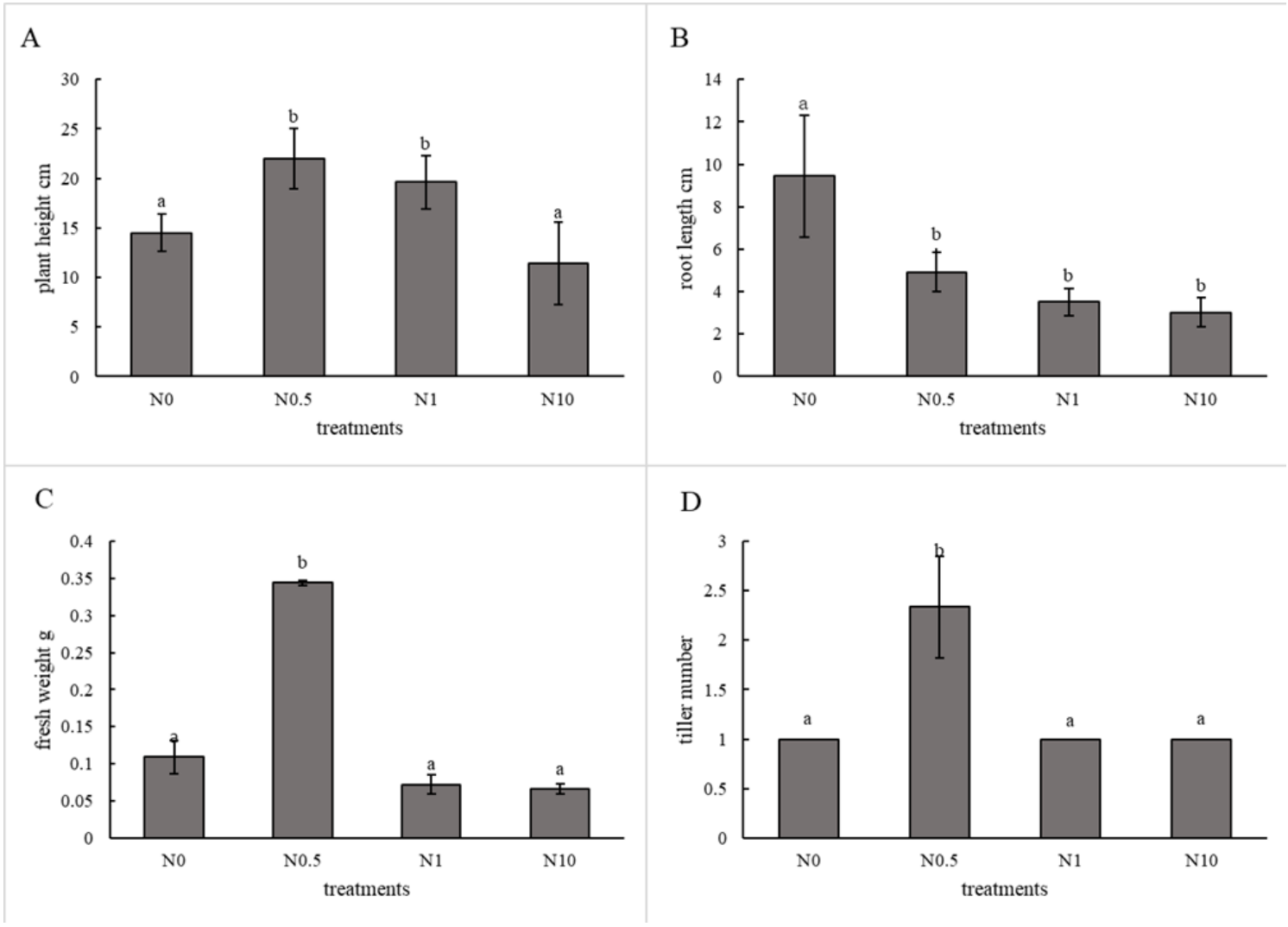

Figure 2

A-D represent plant height, root length, fresh weight and tiller number of different treatments in turn.

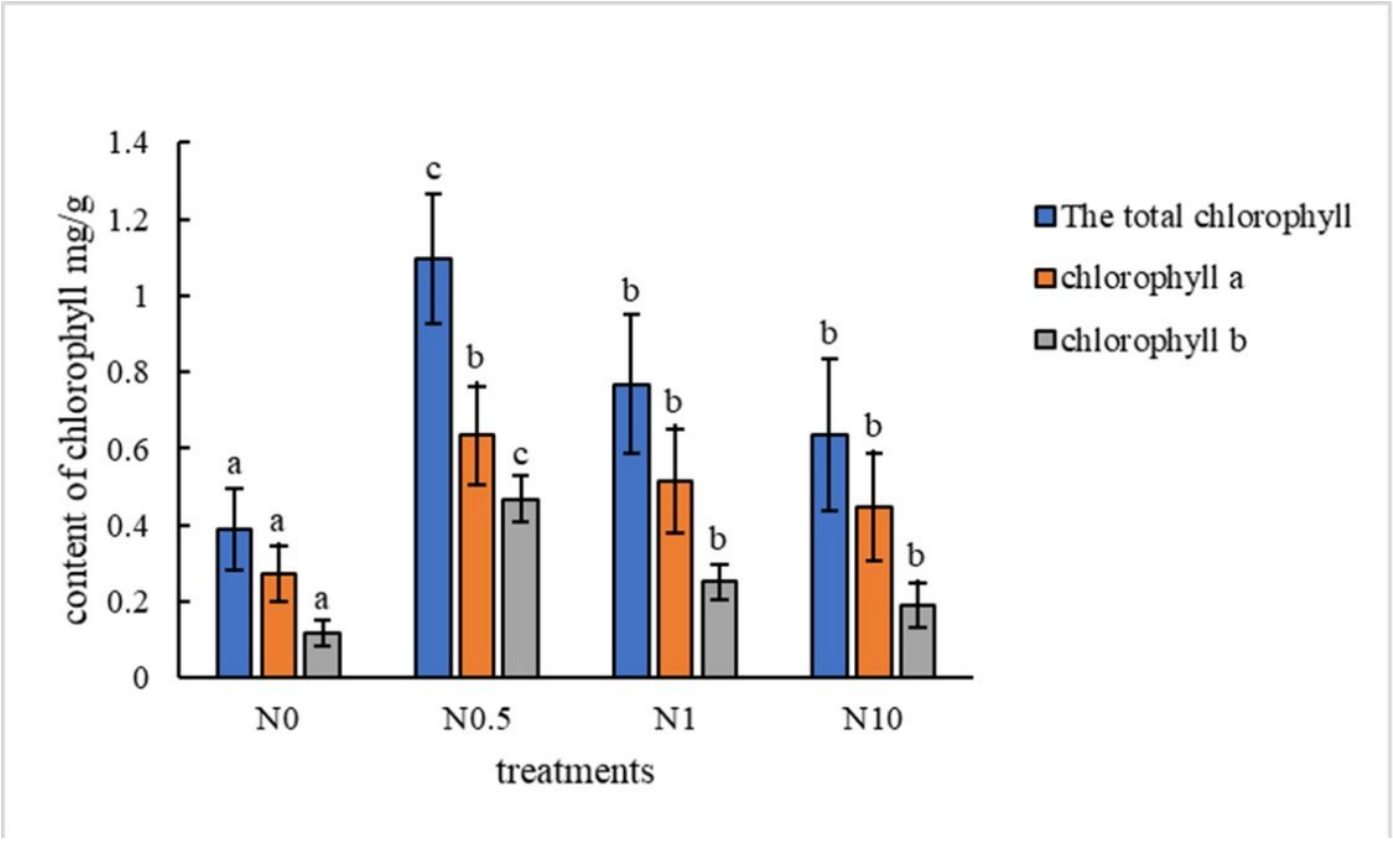

Figure 3

The chlorophyll content. The blue pillars indicate the total chlorophyll content. The yellow and gray pillars indicate the chlorophyll a and b contents, respectively. a and b in all figures represent significant differences, and error bars indicate differences between biological replications.

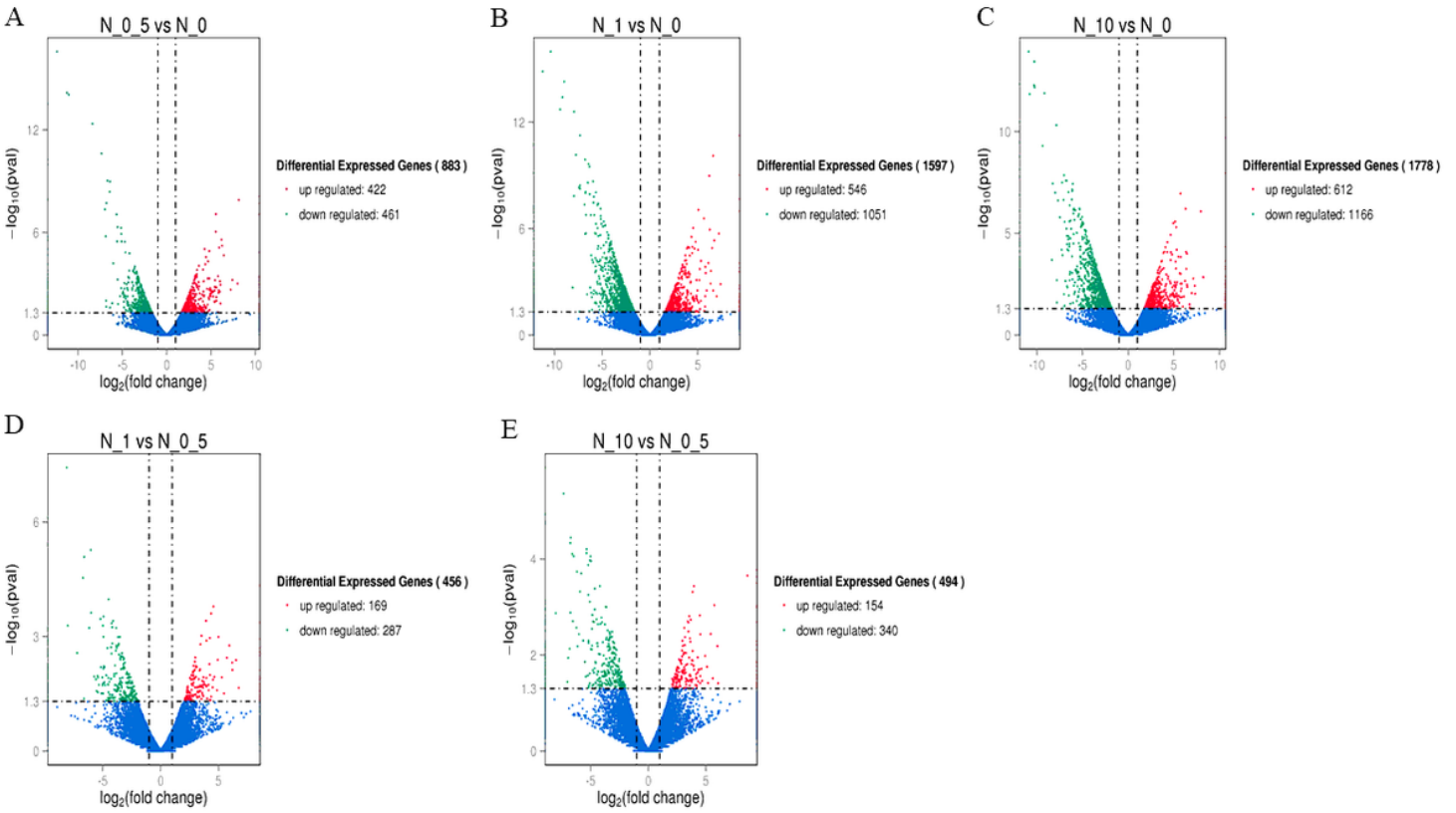

Figure 4

DEG volcano map. A-C represent DEGs in three treatments (N0.5, N1 and N10) with treatment N0 as a control. D-E represent DEGs in two treatments (N1 and N10) with treatment N0.5 as a control. Genes with significant differential expression are represented by red dots (upregulated) and green dots (downregulated), while genes without significant differential expression are represented by blue dots. The abscissa represents the multiple changes in gene expression in different samples. The ordinate represents the statistical significance of the difference in gene expression.

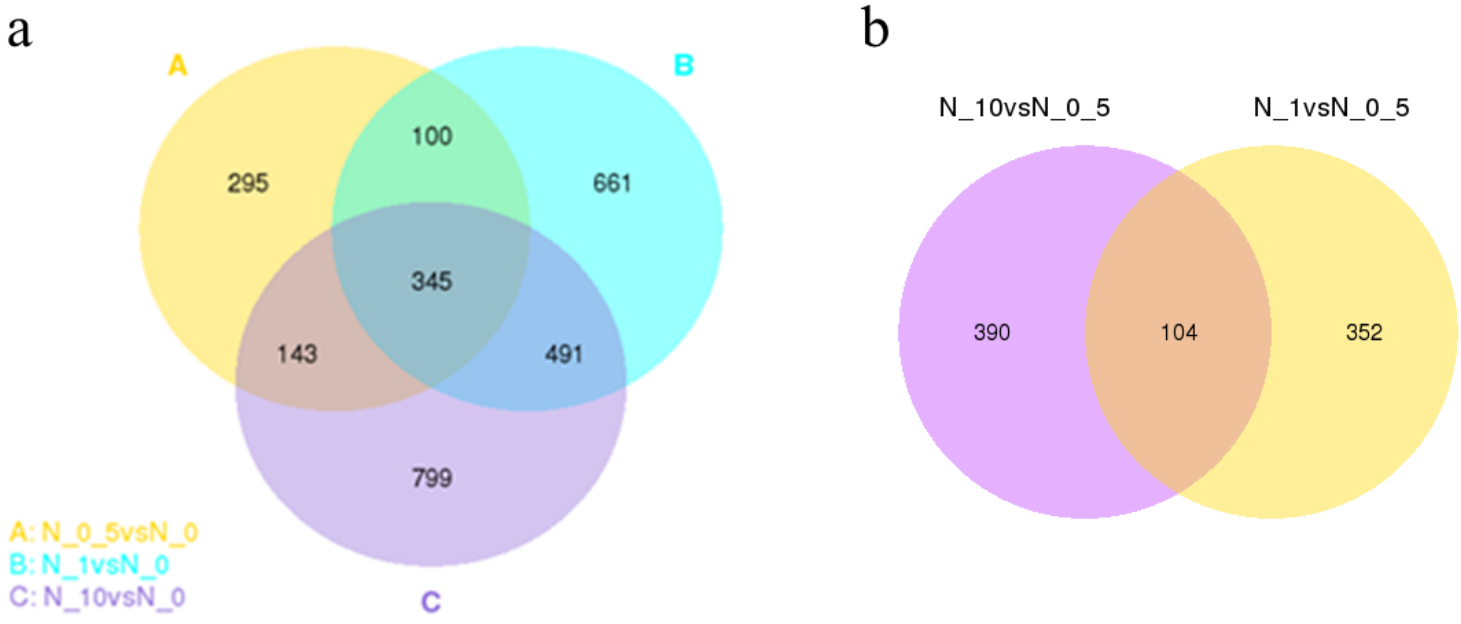

Figure 5

Venn diagram of DEGs. a represents DEGs in three other groups compared to treatment N0 as a control. b represents DEGs in trentments N1 and N10 compared to treatment N0.5 as a control. The sum of the numbers in each large circle represents the total number of differentially expressed genes in the comparison combination, and the overlapping circles represent the number of differentially expressed genes in common between the combinations.

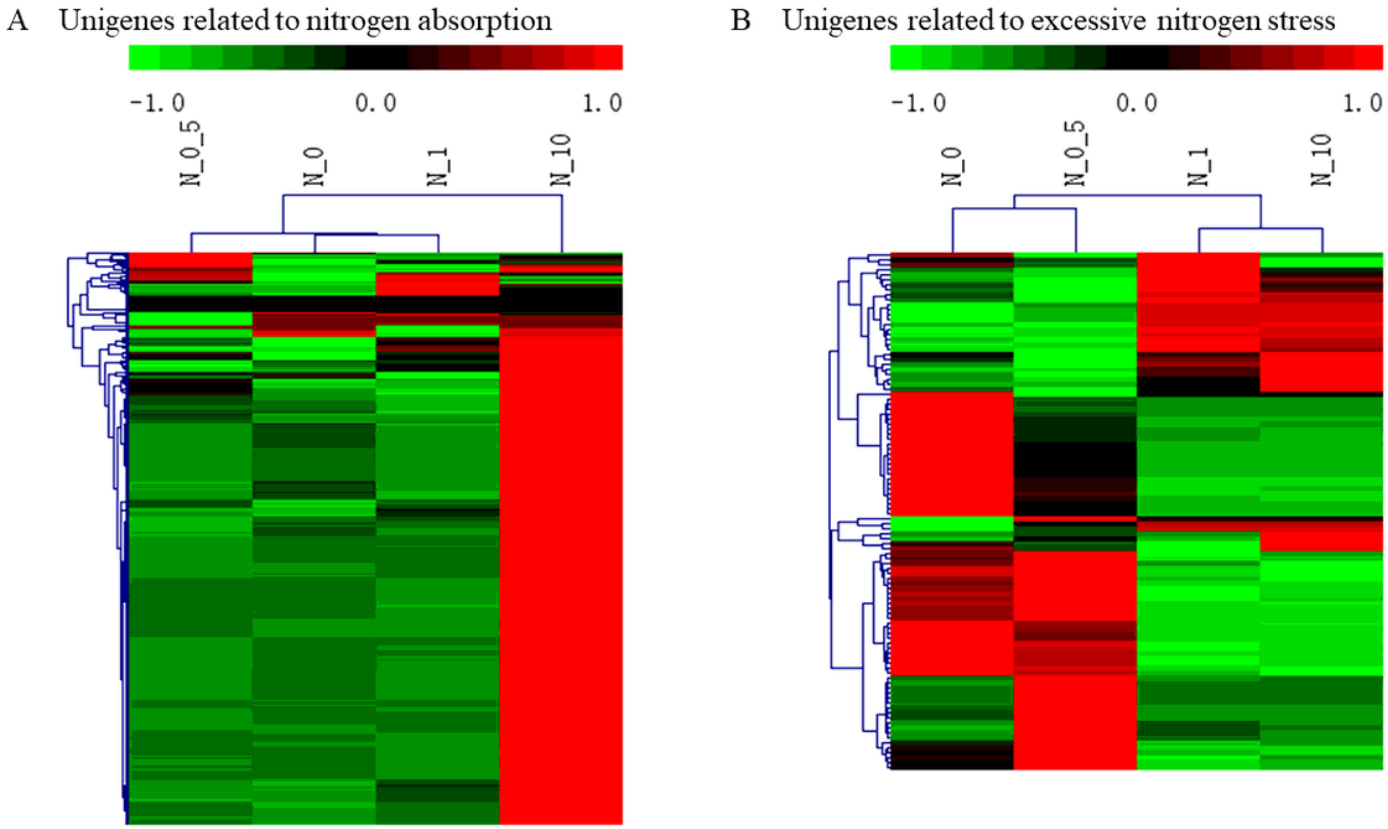

Figure 6

A and B represent the clustering situation of unigenes related to nitrogen utilization and excessive nitrogen stress, respectively. High expression genes are shown in red, and low expression genes are shown in green.

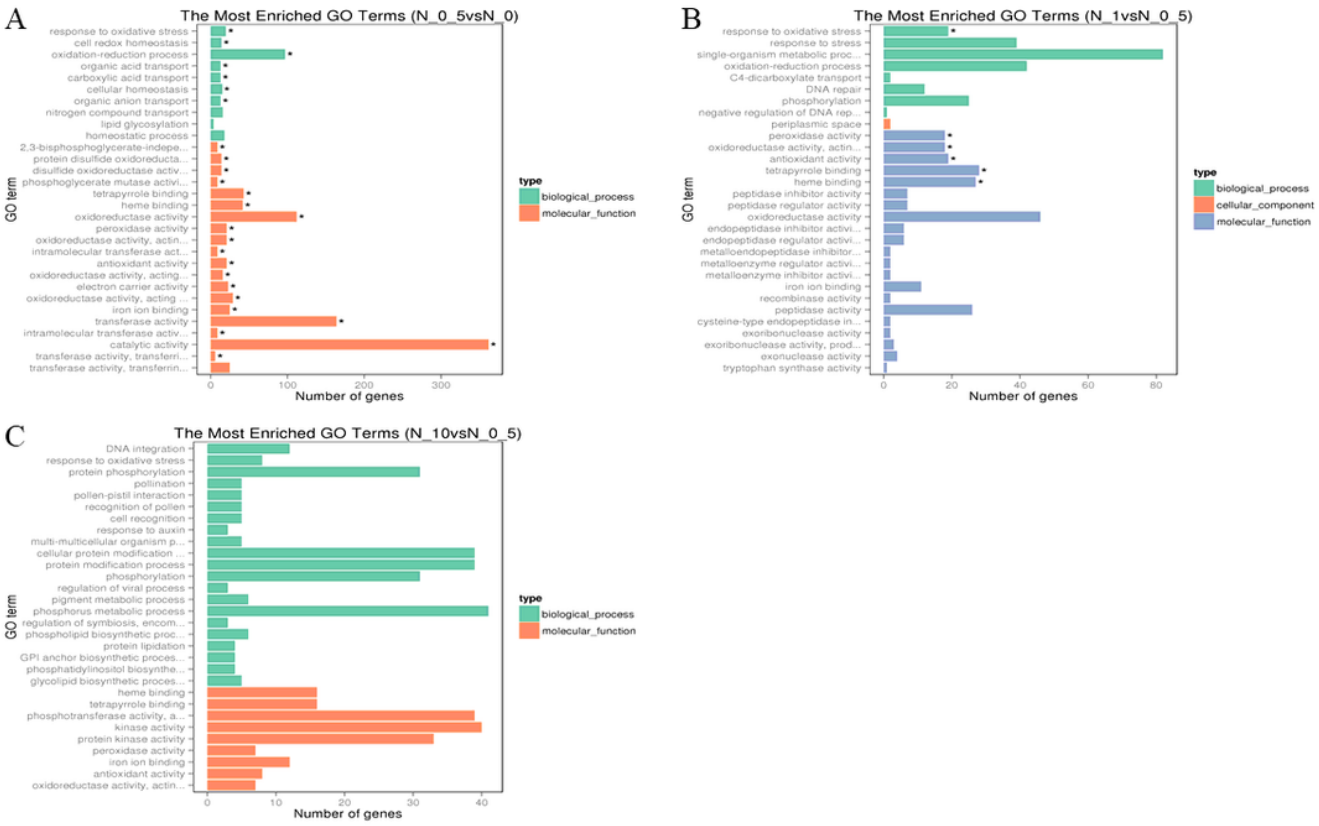

Figure 7

GO enrichment analysis of DEGs. A represents GO enrichment of DEGs in treatment N0.5 compared to treatment N0 as a control. B-C represents GO enrichment of DEGs in the treatment N1 and N10 compared to treatment N0.5 as a control. The ordinate is the enriched GO term, and the abscissa is the number of differentially expressed genes in the term. Different colors were used to distinguish biological processes, cell components and molecular functions, and * was a significantly enriched GO term.

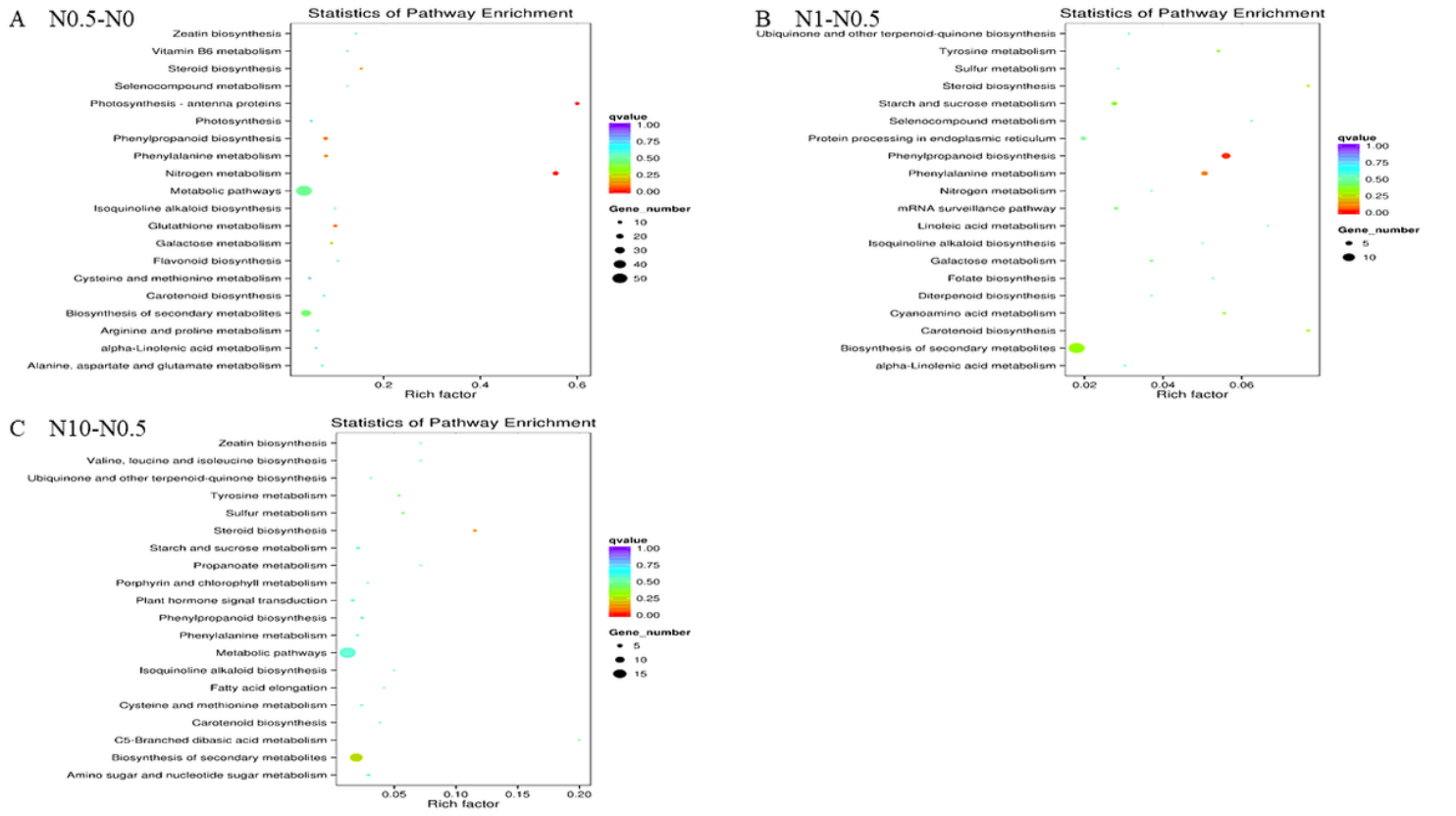

Figure 8

KEGG enrichment distribution of DEGs. The vertical axis represents pathway name, the horizontal axis represents Rich factor, the size of the dots represents the number of differentially expressed genes in the pathway, and the color of the dots corresponds to different Q-value ranges.

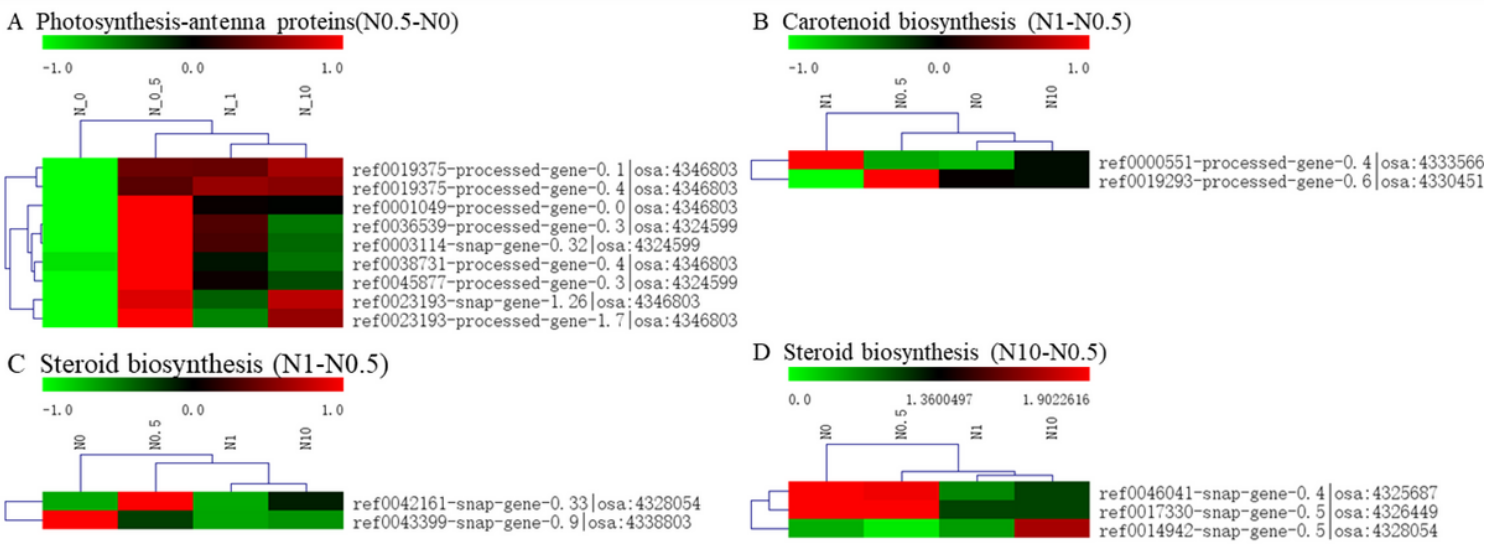

Figure 9

A represents the clustering situation of DEGs enriched in the photosynthesis-antenna protein pathway in treatment N0.5 compared to treatment N0. B-C represents the clustering situation of DEGs enriched in the steroid biosynthesis pathway and carotenoid biosynthesis pathway in treatment N1 compared to

treatment N0.5. D represents the clustering situation of DEGs enriched in the steroid biosynthesis pathway in treatment N10 compared to treatment N0.5. High expression genes are shown in red, and low expression genes are shown in green.

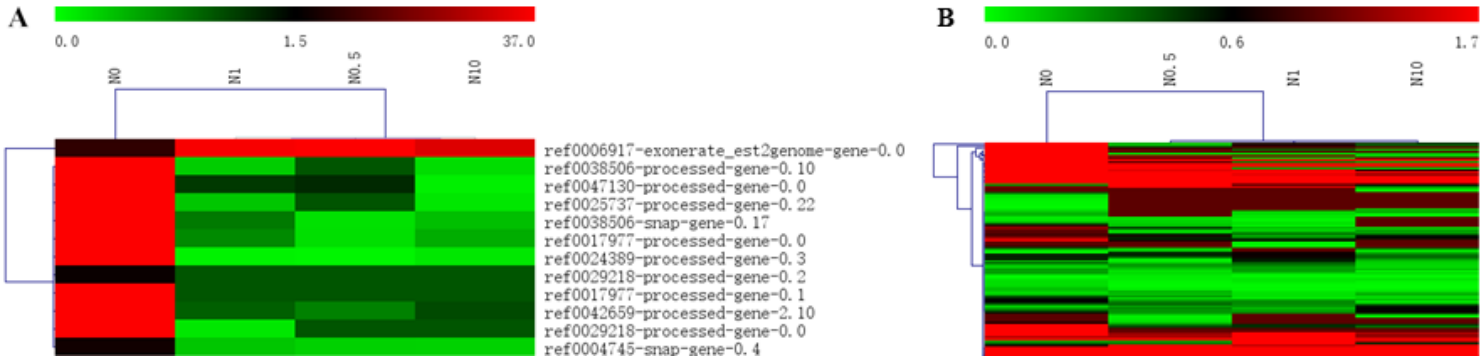

Figure 10

A represents the clustering situation of DEGs enriched in nitrogen metabolism pathway. B represents the clustering situation of DEGs, classified into nitrogen transporter gene family. High expression genes are shown in red, and low expression genes are shown in green.

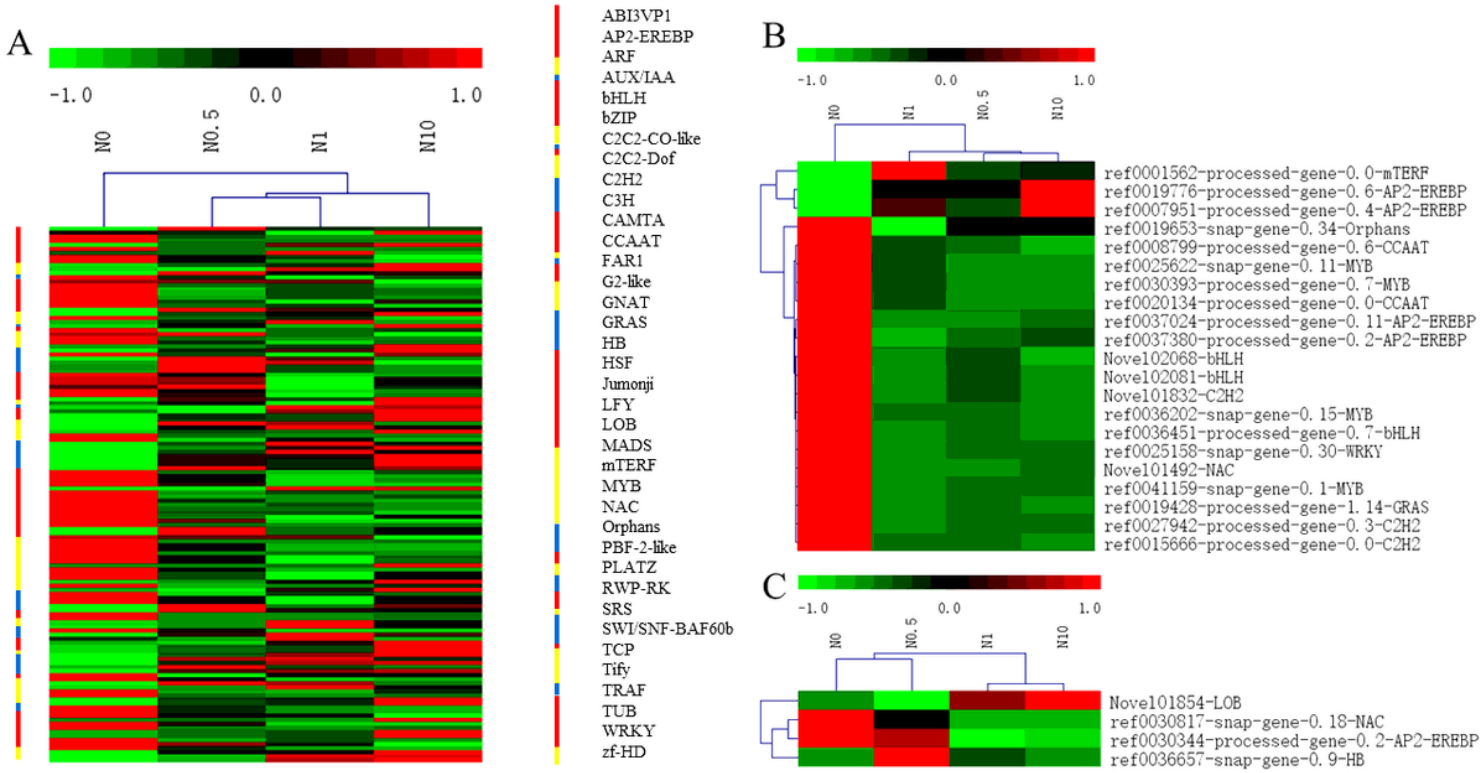

Figure 11

A represents the clustering situation of TF responses to nitrogen in perennial ryegrass. B represents the clustering situation of TFs related to nitrogen utilization. C represents the clustering situation of TFs related to excessive nitrogen stress. Color pillars indicate the different transcription factor families in turn,

and their length means the number of TFs. High expression genes are shown in red, and low expression genes are shown in green.

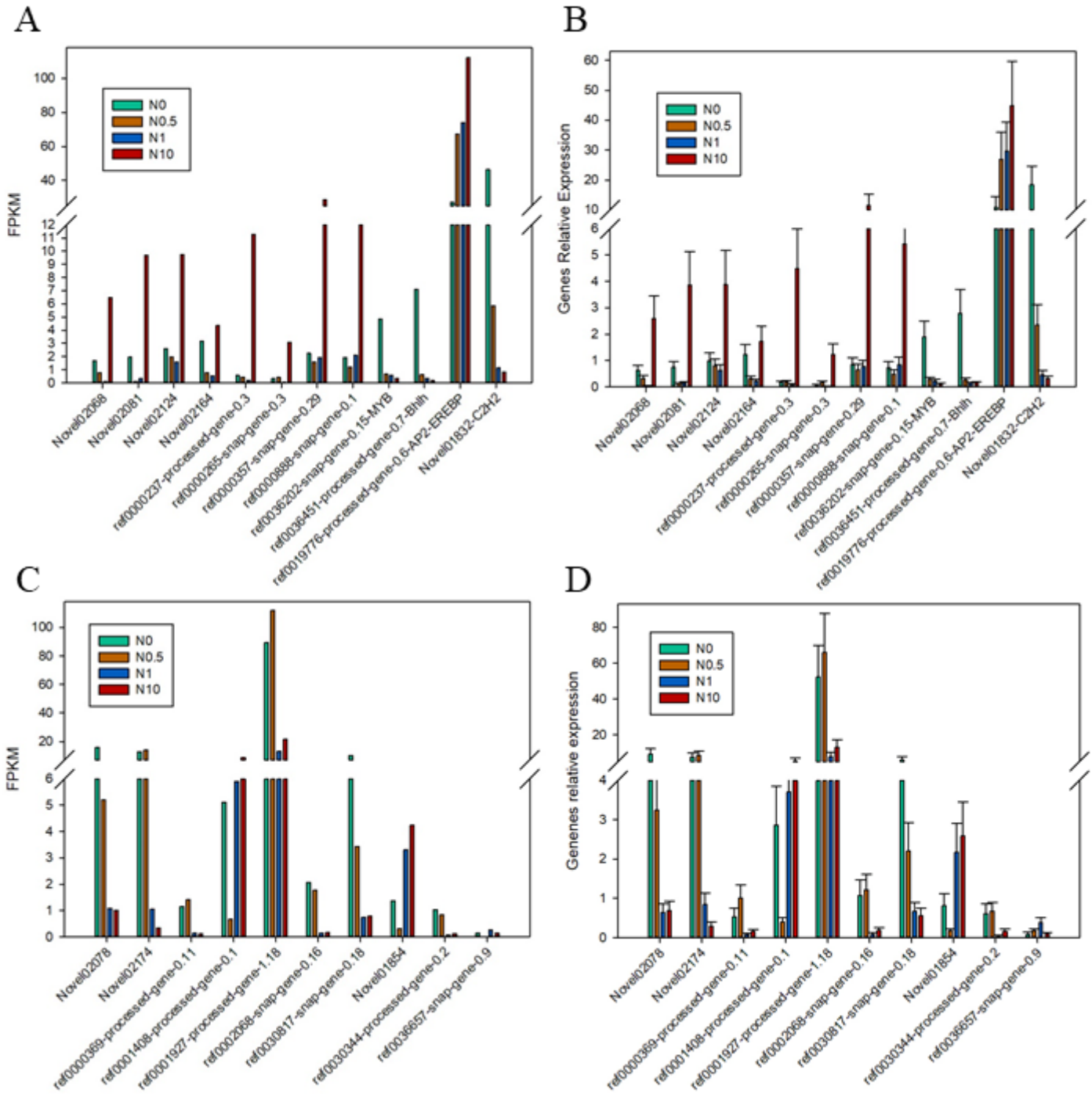

Figure 12

Expression of genes selected for validation. A and C represent FPKM of genes related to nitrogen utilization and excessive nitrogen stress by RNA-Seq. B and D represent the relative expression of genes related to nitrogen utilization and excessive nitrogen stress by qRT-PCR.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplementarymaterials.pdf